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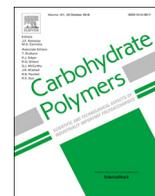
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## Challenging the putative structure of mannan in wheat (*Triticum aestivum*) endosperm



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### ABSTRACT

In wheat endosperm, mannan, is poorly documented. Nevertheless, this hemicellulosic polysaccharide might have a determinant role in wheat grain development since, in *Arabidopsis thaliana*, mutants with a reduced amount of mannan show an altered seed development. In order to gain knowledge about mannan in wheat, we have determined its biochemical structure in wheat endosperm where mannose content is about 0.2% (dry weight basis). We developed a method of enzymatic fingerprinting and isolated mannan-enriched fractions to decipher its fine structure. Although it is widely accepted that the class of mannan present in grass cell walls is glucomannan, our data indicate that, in wheat endosperm, this hemicellulose is only represented by short un-substituted chains of 1,4 linked D-mannose residues and is slightly acetylated. Our study provides information regarding the interactions of mannan with other cell wall components and help to progress towards the understanding of monocot cell wall architecture and the mannan synthesis in wheat endosperm.

### 1. Introduction

The cell wall polysaccharides of wheat (*Triticum aestivum* L.) grain have decisive impacts on wheat end-use properties (e.g. for milling, bread making, starch extraction and brewing) (Courtin & Delcour, 2002; Fincher & Stone, 1986; Saulnier, Guillon, Sado, Chateigner-Boutin, & Rouau, 2013). These cell wall polysaccharides are the main constituents of dietary fibre. Whereas they can have a detrimental effect on animal nutrition – this is often observed for poultry (Scheller & Ulvskov, 2010; Yacoubi et al., 2018) -, cell wall polysaccharides have major beneficial effects on human health (Gómez, Míguez, Yáñez, & Alonso, 2017; Tester & Al-Ghazzewi, 2016).

Cell walls represent less than 5% of the starchy endosperm in wheat. Dominant cell wall polysaccharides have long been reported to be arabinoxylan (AX) then mixed-linkage  $\beta$ -glucan (MLG), but recently cellulose content has been revised and the relative proportion of the different polymers is 60–65% for AX, 20–22% for cellulose and 15% for

MLG, with about 4–7% of mannan (or heteromannan) (Gartaula et al., 2018). The structure and function of AX and MLG have been well-characterized (for a review, see Saulnier, Guillon, & Chateigner-Boutin, 2012). By contrast, both the structure and function of mannan remain to be elucidated. Mannan is a class of hemicelluloses that encompasses: linear mannan and galactomannans with a backbone exclusively made of  $\beta$ -1,4-linked mannose residue, and glucomannans and galactoglucomannans with backbones made of non-repeated pattern of mannose and glucose (Scheller & Ulvskov, 2010). Substitution by  $\alpha$ -1,6-linked galactose on mannosyl residues seems to prevent aggregation of the polymer (Pitkänen, Tuomainen, Mikkonen, & Tenkanen, 2011). The mannosyl residue can be *O*-acetylated at the *O*-2 and *O*-3 positions and, when present, these acetyl groups confer solubility to mannan in aqueous solution (Gómez et al., 2017). Moreover, it is speculated that these acetylations act as a shield against cell wall degrading enzymes (Arnling Bååth et al., 2018; Manabe et al., 2011). Mannan shows a wide spread distribution in plants (for details about the different types of mannans

**Abbreviation:** °DAF, degree days after flowering; AGP, arabinogalactan protein; AX, arabinoxylan; CAZY, carbohydrate active enzymes database; DMSO, dimethyl sulfoxide; EtOH, ethanol; GC, gas chromatography; GH, glycosyl hydrolase; HPAEC, high performance anion exchange chromatography; HPSEC, high performance size exclusion chromatography; Man, Mannose; MLG, mixed linkage glucan; MS, mass spectrometry; NMR, nuclear magnetic resonance; PE, Polymer extract; RT, room temperature; TFA, trifluoroacetic acid

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among plant species and their occurrence in different plant organs see the reviews of [Moreira & Filho, 2008](#); [Liepman & Cavalier, 2012](#); [del Carmen Rodriguez-Gacio, Iglesias-Fernández, Carbonero, & Matilla, 2012](#)). Only two studies provide information regarding the structure of mannan in wheat endosperm. Based on the whole neutral sugar composition of several enriched-cell wall extracts, [Mares and Stone \(1973\)](#) have suggested that cell walls of wheat endosperm contain glucomannan. Recently, [Gartaula et al. \(2018\)](#) have supported this hypothesis through their sugar composition and linkage analysis of enriched-cell wall extracts. In an effort to better understand the relation between the structure and the function of mannan in wheat endosperm, we have characterized its fine biochemical structure. We have developed enzymatic fingerprinting assays, isolated a water soluble mannan-enriched fraction from wheat endosperm and determined the structure of the polysaccharide by combining techniques that included enzymatic digestion, methylation analysis and  $^1\text{H}$  NMR. Our results do not indicate the presence of galactose and glucose residues in the mannan structure of wheat endosperm but instead suggest that, in this tissue, mannan is made of short linear chains of  $\beta$ -1,4-mannose residues.

## 2. Material and methods

### 2.1. Plant material

Wheat (*Triticum aestivum* L.) cultivars Soissons and Recital were used and white flours (containing mainly endosperm tissues) were obtained using a Bühler MLU-202 laboratory mill.

Developing grains (cultivars Recital and SxB049) were grown in containers filled with soil at INRA Clermont-Ferrand (France) in 2012 under natural field conditions until flowering, then they were placed in a growth tunnel (settings 18 °C day /14 °C at night). Grains were harvested at different developmental stages, calculated in Celsius degrees days after flowering (°DAF) using the thermal time method ([Saiyed, Bullock, Sapirstein, Finlay, & Jarvis, 2009](#)). Grains were manually dissected to collect the endosperms.

Reference's mannan polymers from *Amorphophallus konjac* (konjac), *Ceratonia siliqua* (carob) and *Philodendron* (Congo) were either obtained commercially (Megazyme, Bray, Ireland) or kindly gifted. The neutral sugar composition of wheat extracts and reference's mannan is given in [Table 1](#). (1,4)- $\beta$ -D-manno-oligosaccharides (DP 1–6) and galactomanno-oligosaccharides from carob were obtained from Megazyme. Plant material was stored at room temperature (RT).

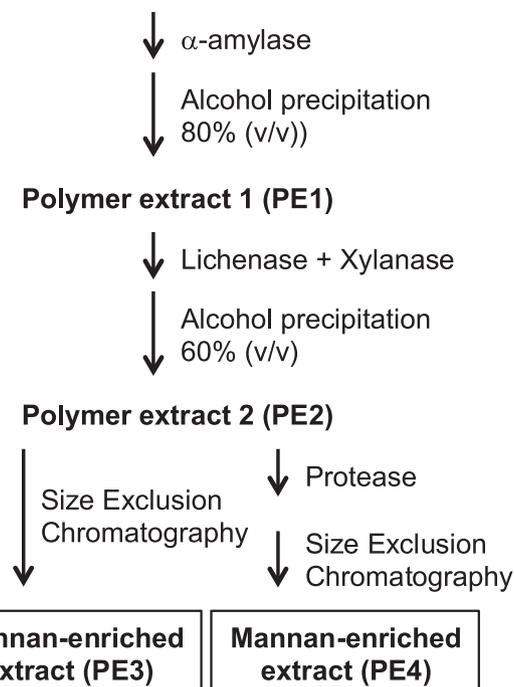
### 2.2. Isolation of a water soluble mannan enriched fraction

Water-soluble mannan was isolated from a water-soluble extract of wheat flour obtained as described in [Faurot et al. \(1995\)](#). The procedure of mannan-enrichment is summarized in [Fig. 1](#).

**Table 1**  
chemical composition of the samples.

| Plant material            | Monomer (% mol) |     |      |      |      |      |      | Sugar content (%weight) |
|---------------------------|-----------------|-----|------|------|------|------|------|-------------------------|
|                           | Rha             | Fuc | Ara  | Xyl  | Man  | Gal  | Glc  |                         |
| Whole wheat grain         | 0.4             | 0.0 | 4.2  | 8.1  | 0.2  | 0.5  | 86.6 | 74.8                    |
| Wheat flour               | 0.4             | 0.0 | 1.5  | 2.7  | 0.1  | 0.3  | 95.0 | 83.0                    |
| Wheat flour water extract | 0.1             | 0.0 | 35.7 | 34.3 | 1.8  | 19.6 | 8.5  | 45.3                    |
| PE1                       | 0.0             | 0.0 | 34.9 | 39.7 | 1.9  | 17.0 | 6.6  | 74.0                    |
| PE2                       | 0.0             | 0.0 | 32.6 | 10.2 | 24.0 | 30.5 | 2.7  | 32.7                    |
| PE3                       | 0.0             | 0.0 | 14.7 | 7.8  | 50.6 | 16.6 | 10.3 | 41.3                    |
| PE4                       | 0.3             | 0.2 | 9.3  | 5.5  | 77.1 | 5.2  | 2.3  | 34.0                    |
| Congo extract             | 0.1             | 0.1 | 0.6  | 0.2  | 97.1 | 0.8  | 1.2  | 74.3                    |
| Carob extract             | 0.1             | 0.0 | 1.4  | 0.4  | 76.5 | 19.8 | 1.8  | 76.4                    |
| Konjac extract            | 0.2             | 0.0 | 0.4  | 0.1  | 61.4 | 0.4  | 37.6 | 95.6                    |

### Wheat flour water-soluble extract



**Fig. 1.** Sequential extraction and fractionation of mannan-polymers from water-soluble extract of wheat flour.

Water-soluble extract from wheat flour (60 g dry material) was dissolved in 1.5 L of distilled (MilliQ) water at 60 °C for 2 h before centrifugation at 6800 g. The supernatant was incubated with 12 U.mL<sup>-1</sup>  $\alpha$ -amylase from *Bacillus licheniformis* (CAZy family: GH13) (Megazyme) at 95 °C for 1 h. Polymer extract (PE) was added with 5 volumes of 95% EtOH (final EtOH concentration 80%) and precipitated overnight at 4 °C. The pellet was recovered by centrifugation rinsed with 80% (v/v) EtOH and centrifuged again. The pellet was blended with 96% EtOH and recovered by filtration through a sintered glass (16–40  $\mu\text{m}$ ) washed with 96% EtOH then with acetone and finally air-dried at 40 °C. The resulting material (PE1) weighted 31.8 g. 10 g of PE1 was incubated overnight with 40 U *Clostridium thermocellum* beta-1,3(4)-glucanase 16A (CAZy family: GH16) (Nzytech, Lisbon, Portugal) and 184 U *Trichoderma viride* beta-xylanase M1 (CAZy family: GH11) (Megazyme) in 200 mL of distilled water at 40 °C. The supernatant was centrifuged, added with 2 volumes of 96% EtOH (final EtOH concentration 60%) and precipitated at 4 °C for 4 h. The pellet was recovered by centrifugation rinsed with 60% (v/v) EtOH, then rinsed and centrifuged with 96% EtOH (2 times) and with acetone (one time). The resulting pellet was dried at 40 °C overnight, then dissolved in water and freeze-dried to give PE2 (989 mg). PE2 was suspended in 1 mL of distilled water and filtered through a 0.45  $\mu\text{m}$  filter (Millex-Hv, PVDF, Millipore, St Quentin en Yvelines, France) and loaded into on Sephacryl S200 HR (GE Healthcare, Uppsala, Sweden) column (3 x 130 cm; V<sub>0</sub>: 57 mL, V<sub>T</sub>: 153 mL) eluted with H<sub>2</sub>O at 1 mL/min. Fraction PE3 was collected from 96 to 144 mL (Kav: 0,41 - 0,9). In a parallel experiment, PE2 (100 mg) was incubated in 1 mL of distilled water containing 35 U protease (Subtilisin A) from *Bacillus licheniformis* (Megazyme) at 40 °C for 3 h. The solution was then heated at 95 °C for 5 min then filtered through a 0.45  $\mu\text{m}$  filter and injected into the Sephacryl S200 HR column as described above. Fraction PE4 (22.5 mg) was collected from 94 to 118 mL (Kav: 0.38 - 0.63). Each PE has been freeze-dried for storage.

### 2.3. Enzymatic fingerprinting with endo- $\beta$ -1,4-D-mannanase

Prior to enzymatic degradation, plant material (100 mg) was suspended in 1 mL of 80% EtOH, incubated at 95 °C for 5 min with vigorous shaking and then centrifuged at 6800 g for 8 min. The pellet was re-suspended in 80% EtOH at RT with vigorous shaking prior centrifugation at 6800 g for 8 min. After repeating these last steps two more times with 96% EtOH, the pellet was dried overnight in a vacuum oven at 40 °C.

Alcohol insoluble residues (AIR) (100 mg) were suspended in 1 mL of distilled water and incubated at 40 °C overnight with 20 U.mL<sup>-1</sup> of a recombinant endo- $\beta$ -1,4-D-mannanase from *Cellvibrio japonicus* (E-BMACJ, Megazyme) (CAZy family GH26). The solution was then heated to 95 °C for 10 min to inactivate the enzyme and then centrifuged at 13,000 g. The supernatant was added with 2 volumes of 96% EtOH and left at 4 °C for 4 h. The 60% EtOH solution was centrifuged at 13,000 g for 15 min at RT, 0.2 mL of supernatant was diluted with 1.8 mL water then filtered through a 0.45  $\mu$ m filter and injected into a HPAEC system for enzymatic profiling.

PE extracts were solubilized in 1 mL of distilled water and incubated at 40 °C overnight with the recombinant endo- $\beta$ -1,4-D-mannanase. Supernatants were filtered through a 0.45  $\mu$ m filter and injected into a HPAEC system for enzymatic profiling.

The oligosaccharides were analyzed with a Dionex Carbo-Pac PA1 column (4  $\times$  250 mm, Thermo Fisher Scientific, Sunnyvale, USA) at 30 °C eluted at 1 mL.min<sup>-1</sup> using a linear gradient of sodium acetate from 0 to 0.2 M in 0.1 M NaOH for 30 min. An ED50 electrochemical detector (Thermo Scientific) was used for detection and the peaks were integrated using Chromeleon software (Thermo Scientific). The retention times of monomers and oligomers released from reference's mannan polymers were used to identify the oligosaccharides released by the endo- $\beta$ -1,4-D-mannanase (See Supplementary Data S1). The samples were analyzed at least twice in separate experiments. Controls for wheat flour were obtained and analyzed similarly, except that addition of endo-mannanase was omitted.

A mass profile of the oligosaccharides generated by endo-mannanase on a wheat flour AIR was obtained by matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) mass spectrometry (MS). An ionic preparation of 2,5-dihydroxybenzoic acid (DHB) and *N,N*-dimethylaniline (DMA) was used as the MALDI matrix, as described in Ropartz et al., 2011. The sample (1  $\mu$ L) was deposited and then covered by the matrix (1  $\mu$ L) on a polished steel MALDI target plate. MALDI measurements were then performed on a rapifleX MALDI-TOF spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam laser (355 nm, 10,000 Hz) and controlled using the Flex Control 4.0 software package. The mass spectrometer was operated with positive polarity in a reflectron mode, and spectra were acquired in the range of 300–2500 *m/z*.

### 2.4. Chemical characterization of cell wall polysaccharides in wheat endosperm

#### 2.4.1. Total neutral sugar and uronic acid content

Total sugar content was measured by colorimetric assay using an autoanalyzer (Skalar, Breda, The Netherlands) (Ray, Vigouroux, Quémener, Bonnin, & Lahaye, 2014). Neutral sugar content was determined through an orcinol/sulphuric acid assay (Tollier & Robin, 1979) where mannose was used as a standard. The uronic acid content was measured using *m*-hydroxydiphenyl along with concentrated sulphuric acid hydrolysis (Blumenkrantz & Asboe-Hansen, 1973) and glucuronic acid was used as a standard.

#### 2.4.2. Neutral sugar composition

Samples (AIR of flour/grains or PE) were hydrolyzed with 2 M sulfuric acid at 100 °C for 2 h or, when analyzing small mass (< 1 mg), with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. Individual

monosaccharides were then converted into alditol acetates (Englyst & Cummings, 1988) and analyzed by gas liquid chromatography as previously described (Yacoubi et al., 2016). The values were obtained from technical duplicate.

#### 2.4.3. Protein content

The protein content was estimated by colorimetric assay (Bradford, 1976) using Bradford reagent (Sigma, Lyon, France) and bovine serum albumin as a standard. The values were obtained from a technical triplicate.

#### 2.4.4. Total carbon and total nitrogen measurements

Total carbon and total nitrogen were measured using an elemental analyser (vario MICRO cube, Elementar Analysensysteme, Hanau, Germany) following an automated dry combustion method (Dumas method) (Thompson, Owen, Wilkinson, Wood, & Damant, 2002).

### 2.5. Structural characterization of mannan from mature wheat endosperm

#### 2.5.1. Analytical high performance size-exclusion chromatography (HPSEC)

Approximately 5 mg of PE were solubilized in 1 mL of distilled water and filtered through a 0.45  $\mu$ m filter. A volume of 50  $\mu$ L was injected into a HPSEC system that consisted of a Shodex OH SB-G guard column (6 mm  $\times$  50 mm; Shodex, Tokyo, Japan) and a tandem of Shodex OH-pak columns SB-805HQ and SB-804HQ (8 mm  $\times$  300 mm). The HPSEC was performed at RT and the columns were eluted at a flow rate of 0.7 mL.min<sup>-1</sup> with 50 mM sodium nitrate. A Viscotek tri-SEC model 270 was used for light scattering and differential pressure detection, and a Viscotek VE 3580 RI detector was used for the determination of polymer concentration while UV was monitored at 280 nm with a LDC/Milton Roy Spectromonitor 3000. A refractive index increment per unit concentration increment (dn/dc) value of 0.146 mL/g was used for concentration determination. Data were collected with Omniseq 4.7 software (Viscotek).

#### 2.5.2. Linkage analysis by gas chromatography-mass spectrometry

The monosaccharide linkage composition was determined using the methylation method described in Buffetto et al. (2015). One mg of dried PE3 was dissolved in 1 mL of dimethyl sulfoxide (DMSO), heated to 80 °C and kept at this temperature until complete dissolution of the sample. The solution was sonicated for 2 min and cooled down prior adding 1 mL of a water-free solution made of ca. 5% (v/v) NaOH in DMSO. Following the addition of 0.5 mL of iodomethane, the solution was vortexed and sonicated for 10 min. The reaction of methylation was stopped by adding progressively 2 mL of distilled water. 2 mL of chloroform was added to the solution which was vortexed prior centrifugation at 6800 g for 5 min. The aqueous phase was discarded and the organic phase was washed three more times by adding each time 4 mL H<sub>2</sub>O prior vortexing and centrifugation. The organic phase was fully evaporated at RT and then hydrolyzed with 2 M TFA at 120 °C for 2 h. The sample were then converted into alditol acetates as described in § 2.4.2. and were injected into a Trace GC Ultra gas chromatograph (Thermo Scientific) mounted with an OV-1 capillary column (length: 30 m, internal diameter: 0.32 mm, column oven temperature 60 °C–315 °C (3 °C/min), carrier gas H<sub>2</sub>) coupled to a ISQ EC single quadrupole mass spectrometer (Thermo Scientific). The data were recorded with Xcalibur software (Thermo Scientific). The samples were tested in duplicate.

#### 2.5.3. <sup>1</sup>H NMR spectroscopy

Prior to NMR acquisitions, 5 mg of PE4 was dissolved in deuterated water to obtain a final concentration of 22 mg.mL<sup>-1</sup>. The NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer equipped with a BBo 5 mm probe. The experiments were recorded at 70 °C to shift the hydrogen deuterium oxide residual peak at 4.3 ppm used as

chemical shift calibration. A quantitative 1D  $^1\text{H}$  spectrum was recorded. A  $^1\text{H}$  90° pulse of 10.7  $\mu\text{s}$  and an accumulation of 256 scans with a recycling delay of 10 s were the more significant acquisitions parameters for the 1D sequence including a water signal presaturation applied to decrease the HDO signal in the same order of magnitude than the others peaks. A 2D homonuclear COSY (CORrelated SpectroscopY) with a presaturation of the HDO signal during relaxation was performed (a  $4096 \times 512$  matrix). 24 scans were accumulated to obtain a sufficient signal to noise ratio. The interpretation of cross peaks in the COSY spectrum was based on previously published data.

### 3. Results

#### 3.1. Mannanase fingerprinting reveals a linear structure for mannan in wheat endosperm

Enzymatic fingerprinting assays were carried out with a recombinant endo- $\beta$ -1,4-D-mannanase from *Cellvibrio japonicus*. A range of commercially available manno-oligosaccharides (Supplementary data S1) was used to identify the manno-oligomers released by the enzyme. We have determined that the minimal number of mannose residues requested for the activity of this mannanase is a trimer (Supplementary data S2A). When incubated with mannopentaose, the enzyme released monomers of mannose and predominantly mannobiose (Supplementary data S2A). The enzyme released about 90% of the mannose present in wheat flour (Supplementary data S2B). The enzyme is therefore suitable to study the structure of mannan for the main population found in wheat endosperm. This was supported by immunoprinting assays where no binding with the LM21 anti-mannan monoclonal antibody (Marcus et al., 2010) was detected when wheat flour was treated with mannanase (Supplementary data S2C).

The enzymatic fingerprint obtained from wheat flour with mannanase was compared (Fig. 2) with that of mannan from congo, glucomannan from konjac and galactomannan from carob (Cescutti, Campa, Delben, & Rizzo, 2002; Katsuraya et al., 2003; Lazaridou, Biliaderis, & Izydorczyk, 2000). The neutral sugar composition of these fractions is shown in Table 1. Only peaks attributed to mannose, mannobiose and mannotriose were identified in the wheat flour extracts treated with the endo- $\beta$ -1,4-D-mannanase. The other peaks were identified as contaminant peaks by comparing the mannanase profiles with profiles of wheat extracts that were not treated with the enzyme (Fig. 2 and Supplementary data S3). The profiles obtained from the wheat flour extracts were similar to that of mannan from congo and distinct to those of konjac glucomannan and carob galactomannan (Fig. 2). The enzymatic fingerprinting thus suggests mannan from wheat flour and thus from mature wheat endosperm is of a linear nature. Moreover, we did not identify peaks that were specifically associated to galactomannan or glucomannan in the extracts of wheat flour (endosperm).

We have analyzed the AIR from dissected endosperms obtained for two different cultivars harvested at five key stages of endosperm development (the stage of: cellularization (150°DAF), differentiation (250°DAF), filling (400°DAF), end of filling (650°DAF), and maturation/desiccation (750°DAF) (Fig. 3). Equivalent results were obtained for both cultivars. For the same amount of AIR extract (50 mg), the peak surface decreased from the earliest developmental stage to the oldest, reflecting the accumulation of starch during endosperm development. However, all the profiles corresponded to that of linear mannan (Fig. 3). By consequence, it suggests that mannan in wheat endosperm is only made of mannose residues regardless of the developmental stage of the grain.

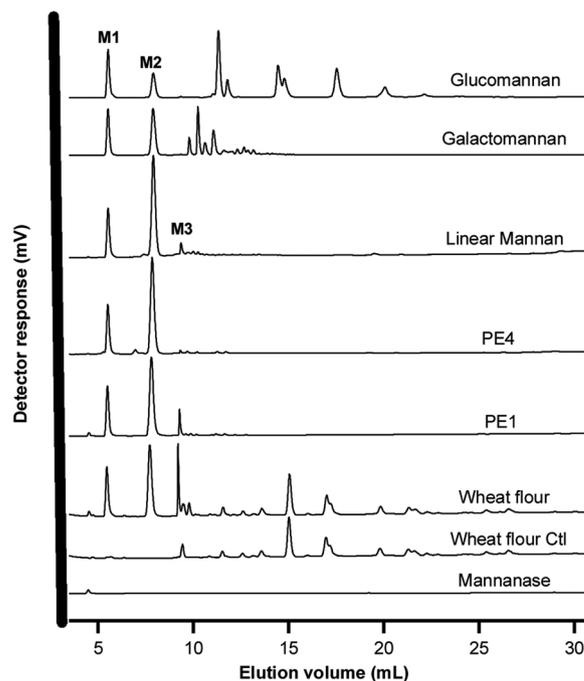


Fig. 2. Mannanase fingerprinting of wheat flour and water soluble mannan-enriched extracts. Samples were injected on a Carbo-Pac PA1 column and elution profiles shown are from top to bottom: glucomannan-enriched extract from konjac, galactomannan-enriched extract from carob, linear mannan-enriched extract from Congo, mannan-enriched extract from wheat flour (PE1 and PE4), wheat flour, wheat flour that was not treated with the enzyme (Wheat flour Ctl), and the mannanase injected on its own (Mannanase). M1, M2 and M3 correspond to mannose, mannobiose and mannotriose peaks, respectively. The area of the mannose peak was used to normalize the different chromatograms with respect to the "wheat flour" sample.

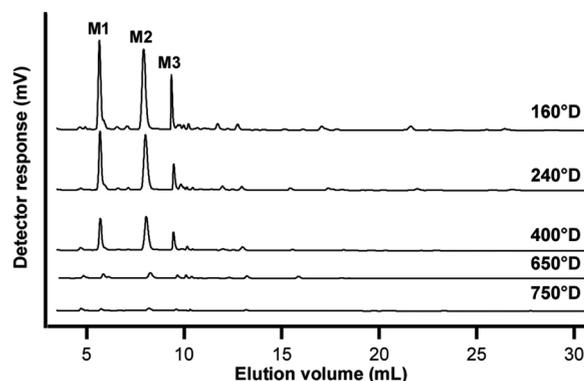


Fig. 3. Mannanase fingerprinting of wheat endosperm extracts harvested at distinct developmental stages (cv SxB049). Samples were injected on a Carbo-Pac PA1 column. The numbers on the right indicate the developmental stage (in °DAF). M1, M2 and M3 correspond to mannose, mannobiose and mannotriose, peaks, respectively.

3.2. The fine characterization of mannan-enriched extracts confirms that, in mature wheat endosperm, mannan are linear chains of  $\beta$ -1,4-mannose residues

#### 3.2.1. Procedure of mannan-enrichment

To confirm the linear nature of mannan in mature wheat endosperm, we have produced and analyzed mannan-enriched PEs from a water soluble extract of wheat flour that is described in Faurot et al. (1995). The polysaccharide fraction of water-soluble extract from wheat flour that is mainly formed of AX, arabinogalactan-proteins (AGP) and MLG consistently contains a small amount of mannose

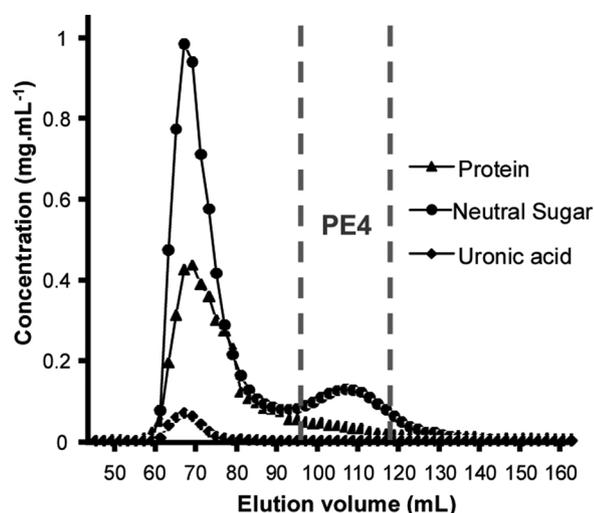


Fig. 4. Size exclusion chromatography on Sephacryl S200 HR of PE2. PE2 was treated with protease then injected on the column and 2 mL fractions were collected. The grey dashed lines delimit fraction PE4 collection.

(Dervilly, Saulnier, Roger, & Thibault, 2000). Depending on wheat cultivars, mannose content determined on AIR of white flour, represents 0.1–0.2% of the flour (dry weight basis, Table 1). The water-soluble extract from wheat flour contains 2% of mannose (Table 1) which account for about 10% of the mannose content present in wheat flour. Although mannan in wheat flour is essentially water insoluble, we took advantage of the presence of the relatively high level of mannose residues in the water soluble extract to attempt the isolation of mannan polymers. The main steps of enrichment are shown in Fig. 1. After successive steps of alcohol precipitations and enzymatic degradations, mannan-enriched fractions were collected from size exclusion chromatography as shown for PE4 in Fig. 4.

The neutral sugar composition of the PEs is presented in Table 1. We have obtained mannose-enriched fractions PE3 and PE4 that contained 50% and 75% of mannose in their total content of neutral sugars, respectively (Table 1). By comparison to the water soluble extract, the yield of mannose for PE1, PE2 and PE4 was of 92.5, 48 and 34%. In the mannanase fingerprinting assays only mannose, manno- and mannose were released from PEs and the proportion of released oligosaccharides was similar to wheat flour (Fig. 2).

### 3.2.2. Physico-chemical characterization of mannan-enriched fractions

Fig. 5 shows that, even though PE4 has been enriched in mannose, the PE is heterogeneous (Fig. 5A and B). Mannan have been co-eluted with proteins and other polysaccharides. Due to this heterogeneity, we have been unable to calculate the molecular weight of mannan-polymers. A comparison of elution profiles between PE4 that were treated or untreated with mannanase highlighted that, in our conditions, mannan-polymers were eluted from ca. 5.6 to 7 mL (Fig. 5A, PE4 vs PE4 + Enz). UV detection at 280 nm confirms that, throughout the procedure of enrichment, the polysaccharides have been co-precipitated and co-eluted with proteins that are resistant to the action of subtilisin A (Fig. 5B). Bradford assays and measurement of the total carbon and nitrogen content indicated PE2, PE3 and PE4 contained 63, 54 and 39% of protein, respectively. As shown in Fig. 5B, the mannanase activity barely affected the elution of proteins within the time frame where mannan-polymers were eluted. This suggests that there is no covalent linkage and no or minor non-covalent interactions with proteins.

### 3.2.3. Structural characterization of mannan-enriched fraction

To assess the linkages of the polymers by permethylation, we used PE3 (Table 2). While terminal mannose and  $\beta$ -1,4-mannose were detected, no other branching was observed for mannose residues in PE3.

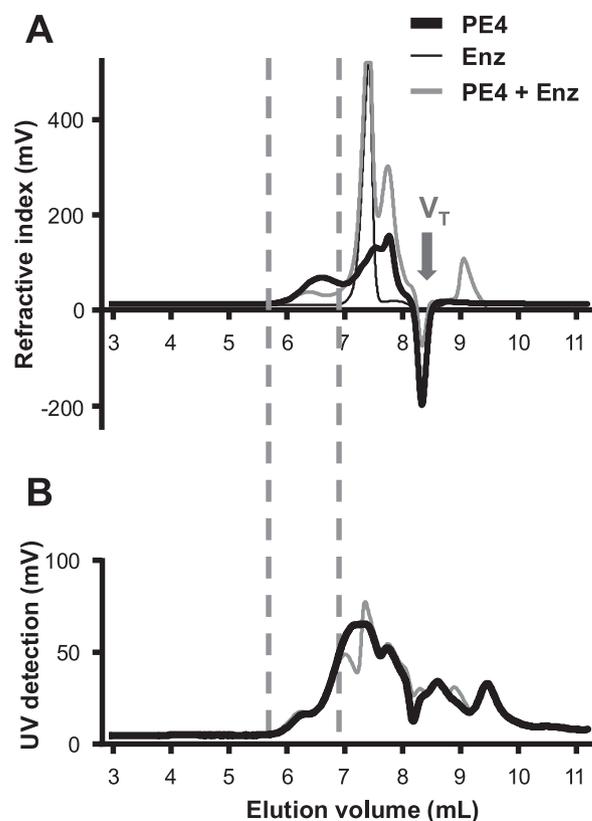


Fig. 5. High performance size exclusion chromatography profile on Shodex OH-pak columns SB-805 HQ and SB-804 HQ of PE4 treated with or without mannanase..

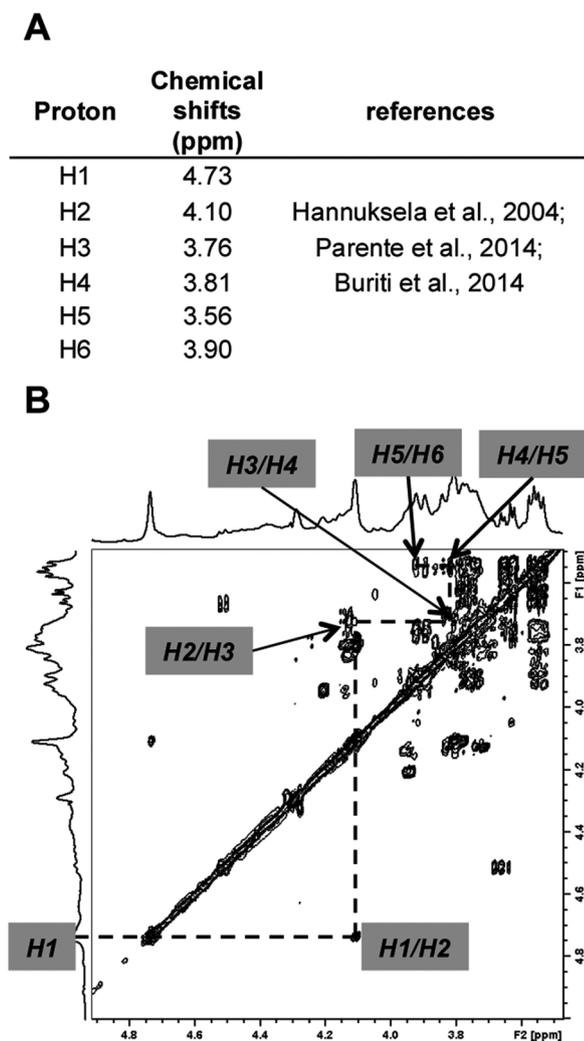
A. The elution profiles of the samples were recorded with a RI detector. The elution profile of the mannanase injected on its own (PE4 -/Enz +) is shown to visualize how the enzyme impacts on the elution profile of the PE4 sample treated with the enzyme.  $V_T$  indicates when the total volume of the columns was eluted. B. The UV detection at 280 nm was recorded to visualize the elution profiles of proteins present in PE4 treated with or without mannanase. The dashed grey lines highlight mannan-polymers elution.

Table 2  
Linkage analysis of PE3.

| Monomer   | Position of methyl group | Linkage    | %Total sugar   |
|-----------|--------------------------|------------|----------------|
| Arabinose | 2,3,5Me                  | terminal   | not determined |
| Xylose    | 2,3 Me                   | 1- > 4     | 2.2            |
|           |                          | 1- > 2,3,4 | 1.9            |
|           |                          | Total      | 4.1            |
| Galactose | 2,4 Me                   | 1- > 3,6   | 7.8            |
| Glucose   | 2,3,6 Me                 | 1- > 4     | 1.1            |
| Mannose   | 2,3,4,6 Me               | terminal   | 2              |
|           |                          | 1- > 4     | 64             |
|           |                          | Total      | 66             |

3,6-Gal as well as traces of  $\beta$ -1,4-glucose were detected. Even though we have detected the presence of terminal arabinose, we have been unable to determine its amount since a contaminant peak was overlapping with the peak of t-Ara. In this experiments, the molar ratio in between t-Man and  $\beta$ -1,4-Man were of 1:32 suggesting a degree of polymerization of 32 and thus a  $M_w$  of 6 kDa. This low  $M_w$  is in concordance with the elution profiles obtained by HPSEC.

To support the results obtained from our methylation analysis, we have analyzed PE4 by  $^1\text{H}$  NMR (Fig. 6). By comparing our spectrum (Fig. 6A) with available libraries of spectra, we only detected the presence of  $\beta$ -1,4-mannose. A deeper analysis by COSY confirmed that  $\beta$ -1,4 is the only linkage detected for mannose residues. We have also



**Fig. 6.** Characterization of PE4 by  $^1\text{H}$  NMR. A. List of the protons detected on the  $^1\text{H}$  NMR spectrum of PE4 that are, according to the literature (Buriti et al., 2014; Hannuksela & Herve du Penhoat, 2004; Parente et al., 2014), associated with mannose residues. B. The COSY spectrum of PE4 confirms that only  $\beta$ -1,4 is the only linkage detected for the mannose residues.

detected traces of acetylated groups in PE4 (Supplementary data S4). In MALDI-TOF MS experiments performed on flour treated with mannanase (Supplementary Data S5), DP3 hexose containing 1 acetyl group ( $\text{Na} + \text{form}$ ;  $m/z$ : 569) was largely detected. Together, these results suggest mannose residues are acetylated.

## 4. Discussion

### 4.1. The structure of mannan in wheat endosperm

Whereas the biochemical structure of AX, MLG and cellulose have been extensively studied in wheat endosperm, the structure of mannan, had never been dissected *in fine*. It is widely admitted that mannan found in grass cell walls are glucomannan (Burton & Fincher, 2014; del Carmen Rodríguez-Gacio et al., 2012; Scheller & Ulvskov, 2010) but, to date, only two studies provided information on the mannan structure present in wheat endosperm. Mares and Stone (1973) have isolated wheat endosperm cell walls through successive steps of fractionation. Based on the monosaccharide composition of cell wall extracts, the authors have concluded that cell wall polysaccharides of wheat are composed of 85% of AX and a 15 remaining percent of MLG and  $\beta$ -glucomannan. Recently, Gartaula et al. (2018) have redefined these

numbers by studying through  $^{13}\text{C}$  CP/MAS NMR and methylation analysis cell wall extracts derived from pure wheat endosperm and milled flour. The authors have provided a complete linkage analysis of cell wall polysaccharides in which mannan is presented as glucomannan that possess a molar ratio of  $\beta$ -1,4-Glu/ $\beta$ -1,4-Man (G/M) that ranges from 1:1 to 1:2 depending of the extract analyzed (Gartaula et al., 2018). The explanation on how the authors attributed the  $\beta$ -1,4-glucose to mannan and how they obtained this ratio is missing from their manuscript which prime focus was cellulose. Indeed, the study from Gartaula et al. have highlighted that cellulose is much more abundant in the endosperm cell walls than what was previously suggested. Here, we have finely characterized the structure of mannan by combining distinct approaches. Altogether, our enzymatic fingerprinting and fine characterization of the mannan structure present in mannan-enriched extracts provide a series of evidence that indicate that mannan in wheat endosperm are short linear chains of  $\beta$ -1,4-mannose residues and are slightly acetylated. In mannanase fingerprinting assays, no manno-oligosaccharide containing galactose or glucose was observed in either the pure wheat endosperm or milled flour or any of its derived-extracts. The mannanase released about 90% of the mannose content of wheat flour essentially in the form of mannose, mannotriose and mannotriose (see Figs. 2 and 3). – Linear mannan are defined as chains of  $\beta$ -1,4-D-mannopyranosyl residues that contain less than 5% of galactose (Moreira & Filho, 2008). To study the mannan structure by enzymatic fingerprinting, our assays relied on the use of a recombinant endo- $\beta$ -1,4-D-mannanase from *Cellvibrio japonicus* (E-BMACJ, Megazyme) (CAZY family GH26) (Hogg et al., 2003). Unlike other enzymes tested in preliminary assays, this mannanase showed no contaminant activity and has an optimal activity at pH 7. Since the enzymatic fingerprinting assays relies on the activity and the efficiency of the enzyme, it could be argued that the elution profiles for the wheat samples are not representative of the whole population of mannan present in wheat endosperm. As pointed above the enzyme releases of about 90% of the mannan present in wheat flour (See Supplementary data S2), furthermore the degradation of extracts from konjac and carob with the endo- $\beta$ -1,4-D-mannanase demonstrated that, in presence of galacto- or glucomannan, the enzyme releases galacto- or gluco-manno-oligosaccharides (Fig. 2 and Hogg et al., 2003). Consequently, we believe that mannanase fingerprinting assays provide an accurate vision of mannan structure in wheat endosperm.

We purified mannan enriched fractions (PE 2–4) from a wheat flour water soluble extract that contained 2% of mannose, rather than from wheat flour that only contain 0.2% of mannose. Although mannose in the water soluble extracts from wheat flour represented only 10% of the wheat flour mannose content, the mannanase fingerprinting of the various PE fractions were similar (Fig. 2) to wheat flour or to linear mannan from Congo fingerprinting. This indicates that mannan in the water soluble extract and in wheat flour cell walls exhibits the same structure. We have successfully obtained PE4 that possessed over than 75% of mannose in its total amount of neutral sugars (Table 1). Although mannose was the most abundant neutral sugar found in PE3 and PE4, the procedure of mannan-enrichment was unexpectedly accompanied by a reduction of the whole polysaccharide content. Indeed, the starting water soluble extract contained 45% (w/w) of polysaccharides whereas PE3 and PE4 possessed 41 and 34% (w/w) of polysaccharides, respectively. Notwithstanding this reduction, the high content of mannose in the polysaccharide fraction of PE3 and PE4 allowed us characterizing the structure of mannan. Small amount of other neutral sugars were still detected in PE3 and PE4. We have concluded that the xyloses present in these PEs belongs to short fragments of xylan that are likely di-substituted by arabinose residues as the xylanase used to degrade AX does not cleave these motifs (McCleary & McGeough, 2015). In our methylation and  $^1\text{H}$  NMR analysis of mannan-enriched extracts obtained from wheat flour (Table 2 and Fig. 6), no  $\alpha$ -1,6-galactose residues were detected. This confirms the absence of branching in the mannan of wheat endosperm. By contrast, both techniques revealed the

presence of  $\beta$ -3,6-Gal, a feature of arabinogalactan II that belongs to arabinogalactan proteins (AGP). Actually the population eluted prior to PE4 on Sephacryl S200 gel (Fig. 4) was essentially constituted of arabinose and galactose (result not shown) indicating the presence of AGP. This was not surprising as our first steps of mannan-enrichment were similar to those Fincher and Stone (1974) carried out to isolate AGP. We have estimated that PE4 contained about 40% of proteins and we assume AGP are present among these proteins. Their resistance to protease and the presence of  $\beta$ -3,6-Gal are two evidence supporting this. It is to note that, while glucuronic acid – another feature of AGP – was present in the peak eluted prior PE4 (Fig. 4), no uronic acid were detected in PE3 and PE4. The AGP present in PE4 are thus expected to be slightly different from those described by Tryfona et al. (2010). A low amount of  $\beta$ -1,4-glucose residues was detected in the linkage analysis of PE3. Interestingly, the molar ratio of  $\beta$ -1,4-Glu/ $\beta$ -1,4-Man (G/M) in PE3 was of 1:2 as observed by Gartaula et al. (2018) in their acid-treated fraction. Seeing that the G/M ratio ranges from 1:1.5 to 1:4.2 in other species (see, for instance: Cescutti et al., 2002; Kato & Matsuda, 1969; Tester & Al-Ghazzewi, 2016; Zhang et al., 2014), a G/M ratio of 1:2 in wheat endosperm could be envisioned. However, by comparison with PE3, narrowing the time frame to collect PE4 from the SEC column did not only result in an increase amount of  $\beta$ -1,4-mannose residues but also in a drastic reduction of the amount of  $\beta$ -1,4-glucose residues (Table 1). This suggests the glucose residues are not covalently linked to mannan residues. In addition, mannanase fingerprinting was quite distinct from that of glucomannan and no peak assigned to glucomannose oligomers were detected in PE or wheat flour samples digested with mannanase. Therefore, we have concluded that these glucose residues belong to other cell wall polysaccharides, possibly cellulose or MLG. Overall, our results strongly suggest the mannan of wheat endosperm are exclusively made of  $\beta$ -1,4-mannose residues. NMR and MALDI TOF MS also revealed the presence of a low amount of acetyl group on wheat mannan.

#### 4.2. The water solubility of mannan and its role in the cell wall architecture of wheat endosperm

The presence of mannan residues in water-soluble extracts from wheat has already been reported (Dervilly et al., 2000). Yet, it is surprising to isolate linear mannan from such extract. Linear mannan found, for example, in ivory nuts form crystalline structure and are insoluble in water (del Carmen Rodríguez-Gacio et al., 2012; Grimaud et al., 2019). Due to the similar structure and their close  $M_w$  - the crystalline mannan found in ivory nuts possess a  $M_w$  ranging from 2.5 to 15 kDa (Moreira & Filho, 2008) and our results suggest the  $M_w$  of linear mannan in wheat endosperm is in the range of 6 kDa -, it would be expected that these polymers display similar chemical properties. However, seeing that the mannanase we used does not hydrolyze crystalline mannan (Hogg et al., 2003), it is unlikely that mannan of wheat endosperm form crystalline structure. Only low amount of acetylation were detected in wheat mannan and, even though we do not exclude it, it is unlikely that acetyl groups prevent the linear mannan to crystallize. The water solubility of mannan in wheat endosperm could indicate they are not located in the cell walls. Mannan has been shown to be stored in the vacuoles of storage tissues in many plants (Yildiz & Oner, 2014) and it is believed that the favorite target of the GH26 endo-mannanase used are storage mannan and manno-oligosaccharides (Hogg et al., 2003). Palmer et al. (2015) have carried out immunomicroscopy studies of wheat endosperm and have shown that mannan are located in the cell walls. Another hypothesis regarding the water solubility of linear mannan in wheat endosperm is that it results from the interactions of the polymer with other cell wall components. No covalent linkage in between mannan and other polymers was detected on the isolated water-soluble fractions. However, there are a remaining 10–15% of mannanose residues that are not released from wheat flour by the endo-mannanase from *C. japonicus*. It is thus possible

that these residues are attached to other cell wall components. All along the procedure of mannan-enrichment, the polysaccharide have been co-precipitated and co-eluted with proteins. However, a comparison of UV detection in between PE treated with or without mannanase (Fig. 5) indicate both polymers are not covalently linked and suggest they barely interact through non-covalent interactions. In *A. thaliana*, cell walls possess macromolecules made of pectins and hemicelluloses that are covalently linked to a core of AGP (Tan et al., 2013). Likewise, cell wall proteins have been shown to bind to cell wall polysaccharides through non-covalent interactions (Hijazi, Roujol et al., 2014; Hijazi, Velasquez, Jamet, Estevez, & Albenne, 2014). By comparison to the Type I walls of *Dicotyledons* (such as Arabidopsis) that features xyloglucan and large amount of pectins, the Type II walls of *Commelinoid Monocotyledons* which is the Clade wheat belong to possess mere portions of pectins and large amount of hemicelluloses that are distinct to those of dicots (for instance, MLG and glucuronarabinoxylan) (Silva et al., 2011). However, both cell wall Types contain in average 10% of proteins (Francin-Allami et al., 2015). By consequent, even though it has not been reported yet, proteo-glycan complex formed through covalent and non-covalent interactions are also expected to be present in monocot cell walls. Our results indicate that the water soluble mannan-polymers characterized in this study can be excluded from such complex. As they share the same conformation, mannan and cellulose can interact through hydrogen bounds and many tight associations in between mannan and cellulose have been reported (Voiniciuc et al., 2015; Whitney, Brigham, Darke, Reid, & Gidley, 1998; Yu et al., 2018). Based on the literature and their results, Gartaula et al. (2018) have presented a cell wall model for the endosperm cell walls of wheat where cellulose and mannan along with arabinoxylan form a scaffold that is embedded in a matrix predominantly composed of AX and MLG but our results are not in favor for this model. Firstly, the mannan-polymers we have isolated from wheat flour are water-soluble which means they are unlikely tethered to cellulose through sturdy covalent or hydrogen bonds. Secondly, mannan-polymers are easily enzymatically degraded in wheat flour. This would not happen if they belonged to an inner scaffold embedded in a matrix of AX and MLG. As mannan account for about 7% of the cell wall polysaccharide, it is unlikely that they act as a filler agent. Instead, we propose that the water-soluble linear mannan present in wheat endosperm act as a spacer in the cell wall architecture. Further studies are required to fully appreciate the impact mannan has in the context of the whole wall architecture of wheat endosperm.

## 5. Conclusion

We have successfully characterized the fine structure of mannan in wheat endosperm. Our data suggests they are made of short chains of unsubstituted  $\beta$ -1,4-mannose residues and are slightly acetylated. This does not corroborate with the hypothesis presented by Mares and Stone (1973) and with the general consensus that the mannan of monocot cell walls is glucomannan (Burton & Fincher, 2014; Scheller & Ulvskov, 2010; del Carmen Rodríguez-Gacio et al., 2012).

Our study provides useful data to progress in the understanding of mannan synthesis of wheat endosperm and allows better integrating mannan in the context of cell wall architecture of monocots.

#### Author contribution

Original idea: YV, ALCB, LS. Experimental work: YV, XF, MS, SLG. Writing of the manuscript: YV, XF, SLG, ALCB, LS.

#### Declaration of Competing Interest

This research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.carbpol.2019.115063>.

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